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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,259,745**, on January 19, 1999, by **UNIVERSITE DE MONTRÉAL,** assignee of Bruno Paquin, Ivan Brukner and Guy Tremblay, for "Generation of Oligonucleotide Libraries Representative of Genomes or Expressed MRNAS (CDNAS) and Use Thereof".

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ABSTRACT OF THE DISCLOSURE

The present invention relates to oligonucleotide librairies and use thereof. In particular, the present invention relates to specific oligonucleotide librairies, the oligonucleotides being of unique length , and to the use thereof. The present invention further relates to the use of these OLs in numerous biotechnological applications including a the identification and/or characterization of biological materials, clinical diagnosis (DNA/RNA level), preparative extraction of specific mRNA (and genes) and genomic research/mapping.

TITLE OF THE INVENTION

GENERATION OF OLIGONUCLEOTIDE LIBRARIES REPRESENTATIVE OF GENOMES OR EXPRESSED MRNAS (CDNAS) AND USE THEREOF

FIELD OF THE INVENTION

The present invention relates to oligonucleotide librairies and use thereof. In particular, the present invention relates to specific oligonucleotide librairies, the oligonucleotides being of unique length , and to the use thereof. The present invention further relates to the use of these OLs in numerous biotechnological applications including a the identification and/or characterization of biological materials, clinical diagnosis (DNA/RNA level), preparative extraction of specific mRNA (and genes) and genomic research/mapping.

BACKGROUND OF THE INVENTION

The generation of genomic DNA libraries, or cDNA libraries and the maintenance, and handling of these libraries are critical procedures in the field of genomics and/or biotechnology. In classical libraries the relevant segments of DNA are cloned into vectors, which are maintained and propagated in particular biological systems (in vivo). Alternatively, libraries (in vitro) can be directly constructed from genomic DNA or cDNA. They contain linkers at the 5' and 3' ends of the DNA which allow PCR amplification of the library. The information stored in these libraries contains repetitive sequence elements that originated from repetitive DNA, or high copy mRNAs. This results in a significant redundancy, which can complicate the use and the outcome of using

classical libraries. Another important feature which reduces the utility of classical libraries is their heterogeneity in size of each member of the library. Since the success of subtractive hybridization procedures is dependent upon the length, complexity and the redundancy of the libraries, the results are particularly sensitive to the choice of method and the number of cycles performed. In fact, one must use gross hybridization conditions that can accommodate the heterogeneous length and redundancy of stored information in order to perform subtraction. Thus, the results are more "laboratory-specific" than library-specific. There thus remains a need for librairies which enable the use of hybridization conditions which are uniform, library-specific, controllable, reproducible with high resolution and are thus highly informative.

Akopyants et al. (Proc. Natl. Acad. Sci. USA 95:13108-13113) performed subtractive hybridization using bacterial DNAs digested by high-frequency restriction enzymes. The use of such a restriction enzyme tends to generate DNA fragments having a broadly similar size, about 500 base pairs. However, the uniformity is not rigorous. Furthermore, such a library of restriction fragments still contains a significant number of redundant sequences. For these reasons, the conditions of hybridization are not very well resolved. In addition, patches of short polymorphism embedded in homologous sequences are going to be missed using such a type of library.

There thus remains a need to provide a type of library which minimizes or eliminates redundant sequences, thereby enabling well resolved hybridization conditions.

The present invention seeks to meet these and other needs.

The present description refers to a number of documents, the content of which is herein incorporated by reference.

SUMMARY OF THE INVENTION

The present invention relates to methods for generating OLs from genomic DNAs and cDNA and to the subtraction of these libraries.

The present invention further relates to oligonucleotide librairies which enable the use of hybridization conditions which are uniform, library-specific, controllable, reproducible with high resolution and are thus highly informative.

In addition, the invention relates to unique length oligonucleotide libraries, which minimize or eliminate redundant sequences, thereby enabling well resolved hybridization conditions.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the invention, reference will now be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and in which:

Figure 1 shows A) Hybridization of random oligonucleotide mixture with template (genomic DNA/cDNA) of biological origin. High fidelity hybridization was done by using stringent hybridization conditions (as detailed in B) or mismatch free ligation procedure (as shown in C), followed by the separation of hybridized from non-hybridized material and amplification of hybridized oligonucleotides. The non-hybridized material is used in the next round of rehybridization, until all complementary sequence motifs are captured. B) General

approach, in which a single oligonucleotide library is hybridized to the target DNA (1) and selected oligonucleotides are amplified by PCR (2).

C) The thermostable ligation-dependent approach, in which two oligonucleotides must hybridize side by side (1) without mismatches for the ligation to occur (the dot indicates the ligated link). As in B), the selected oligonucleotides are amplified by PCR (2).

Figure 2 shows the Subtraction between different oligonucleotide libraries (OL1 and OL2), which do contain common sequence motifs: The first step is composed of streptavidin/biotin-based partitioning of double stranded OL under denaturing conditions to separate: (i) antisense stand (which was hybridized with template) and (ii) sense strand: In the next step, antisense strand of OL1 was mixed under hybridization conditions with an excess of sense strand of OL2. The double stranded mismatch-free hybrids (OL1 antisense/OL2 sense) and single stranded OL2 sense molecules are separated from single stranded antisense OL1 molecules. Finally, the OL1 antisense molecules are amplified and the whole procedure is repeated until none of the OL1 antisense molecules has complementary sequences to any of the OL2 sense molecules.

Figure 3 shows the validation of the Ols in accordance with the present invention.Lambda and adenovirus DNAs were dot blotted on nylon membranes and hybridized with radio-labeled oligonucleotides from the starting random pool, the OL constructed from the lambda DNA or the OL constructed from the adenovirus DNA.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the

accompanying drawing which is exemplary and should not be interpreted as limiting the scope of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention thus provides, oligonucleotide libraries having the following characteristics:

1) Unique length (typically 60 bp, of which the center 20 bp are representative of the library). This unique length allows clear separation of complementary *sense* from *antisense* strands (biotinylated *versus* non-biotinylated strands).
2) Oligonucleotide libraries have a uniform number of copies for each sequence motif. Therefore, there is no differential hybridization kinetics which could originate from the presence of repetitive DNA.
3) As a consequence of 1) and 2), the melting profile of these OL (or hybridization kinetics) has a sharp transition from double stranded to single stranded (or vice versa). This is a critical advantage in subtractive hybridization procedures.

In one particular embodiment, the starting pool of oligonucleotides is chemically synthesized and consists of a random region of a fixed length (L), flanked by a constant sequence (primer binding sites, PBS). The random oligonucleotide pool covers n copies (n=1,2,3...) of all sequence combinations of length L, i.e. 4^L, which is a total of 10^{12} different sequence motifs for L=20 nucleotides. The basic length of oligonucleotides is long enough to generate unique sequence motifs for a particular biological system. The complexity of the library (10^{12}) overcomes the complexity of the template (which is usually between 10^4-10^9). The random pool is used as starting material for

producing the biologically relevant oligonucleotide libraries (from genomic DNAs or cDNA libraries, or subtractive variants). The constant flanking sequences are used to amplify selected oligonucleotides by PCR.

The method uses a) stringent hybridization and/or b) mismatch free hybridization and ligation. High fidelity hybridization between pools of oligonucleotides and templates (genomic DNA or cDNA) is the basic mode of transfer of genomic information into OLs. A new and more efficient subtractive hybridization procedure, which differs from classical cDNA/genomic subtractions, has been developed to accommodate the features of the aforementioned OLs.

The present invention is illustrated in further detail by the following non-limiting examples.

EXAMPLE 1

GENERATION OF OLs, USE THEREOF IN SUBTRACTIVE HYBRIDIZATION TO GENERATE SOLs, AND USE OF OLs OR SOLs IN HYBRIDZATION EXPERIMENTS

OLs are generated from the template DNAs. These OLs are used in subtractive hybridization, for example between genomic or cDNA-based library (OL1 and OL2) to make a new Subtractive Oligonucleotide Library (SOL1/2 and/or SOL2/1)), that is/are specific for one system/library but not for the other. Oligonucleotides isolated from such subtractive libraries (SOL) are useful for diagnostic purposes. They can a) directly serve as highly specific hybridization probes or b) they can be tested for PCR-specific differential amplification, specific for one, but not the other biological system.

These libraries (OL or SOL) can be hybridized to oligonucleotide chip arrays in order to obtain a specific hybridization pattern that is useful for diagnostic features: each OL produces an image which is specific for the templated DNA (genome or cDNA). A particular advantage in using OL or SOL instead of genomic/cDNA libraries is that the hybridization signal is not dependent on copy number and distribution of particular sequence motifs. By comparing images of different genomes/cDNA, one can deduct which oligonucleotides are highly specific for a single genome/cDNA, and use this or these oligonucleotide(s) as "genome tags". The oligonucleotides obtained can also be used for specific diagnostic PCR.

Ols or SOLs can be inferred from two biologically relevant systems, like mammalian cells, to detect fine differences in cell cycle, tissue status, viral infection, age/development status etc.

Experimental facts:

To validate our approach, we first tested the procedure with a simple system. We generated OLs using lambda phage and adenovirus genomes as templates, separately. The complexity of these genomes is around 10^4, which corresponds to a small fraction of the total complexity of the starting random pool ($1/10^8$). Lambda and adenovirus genomes do not have any 20-mer motifs in common, which means that an OL made from lambda DNA should not cross-hybridize to the adenovirus DNA, and *vice versa*. The starting mixture of random oligonucleotides is such that it should contain about 10 copies of each possible 10^{12} different sequences. As shown in Fig. 3A), when the random pool of the oligonucleotides is radio-labeled, both genomes are hybridizing to the probes. However, after construction of high fidelity OLs, the hybridization

signals become specific to the genome from which the OL was inferred (Fig. 3). Therefore, OLs are indeed reflecting the original template.

Second, artificial mixture of template DNAs (lambda and adenovirus) is made in different ratios to mimic conditions of subtractive hybridization. Having lamda OL on the one hand and these different mixtures on the other hand, we are proving that we could subtract the lamda-specific oligonucleotides, and isolate the adenovirus-specific ones. We are then planning to test these concepts on more complex systems, like bacterial genomes (complexity of about 10^6) and, ultimately, mammalian cells (complexity of about 10^9).

Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

WHAT IS CLAIMED IS:

1. A method of generating an oligonucleotide-based library which originates from a chosen biological material, comprising:

a) generating a random oligonucleotide mixture, wherein said oligonucleotides are of a unique length;

b) hybridizing said random oligonucleotide mixture of a) with a nucleic acid template of biological origin under hybridization conditions which enable the formation of duplexes, while minimizing or abrogating mismatches;

c) separating said duplexes from non-duplexed material;

d) amplifying said hybridized oligonucleotides;

e) rehybridizing non-hybridized material with said template;

f) repeting said steps a) to e) until all oligonucleotides that can hybridize have been captured.

2. The method of claim 1, wherein said template is genomic DNA or cDNA.

3. The method of claim 1 or 2, wherein said hybridization conditions are carried out using one of: stringent hybridization conditions, mismatch-free ligation of random oligonucleotides, type 5' primer$(N)_{7-10}$ 3'and 5'$(N)_{7-10}$ primer 3', using thermostable ligases, or a combination thereof, mismatch-free ligation of random oligonucleotides, type 5' primer$(N)_{7-10}$ 3' and 5'$(N)_{7-10}$ primer 3', using thermostable ligases, or combination thereof.

4. The method of claim 1, 2, or 3, wherein said biological material is selected from genomic DNA, and cDNA.

5. A method of subtracting between different oligonucleotide libraries which contain common sequence motifs comprising:

a) a partitioning of double stranded OL under denaturing conditions to separate: (i) antisense strands hybridized with template and (ii) sense strands;

b) annealing said antisense strand of OL1 with an excess of OL2 sense strand, under hybridization conditions;

c) partitioning of double stranded mismatch-free hybrids (OL1 antisense/ OL2 sense) and single stranded OL2 sense molecules from single stranded antisense OL1 molecules; and

d)amplification of OL1 antisense molecules and repetition of steps a) to d) until OL1 antisense does not have complementary sequences to OL2 sense.

6. The method of claim 5, wherein said partitioning is carried out using streptavidin/biotin.

Fig 1 A

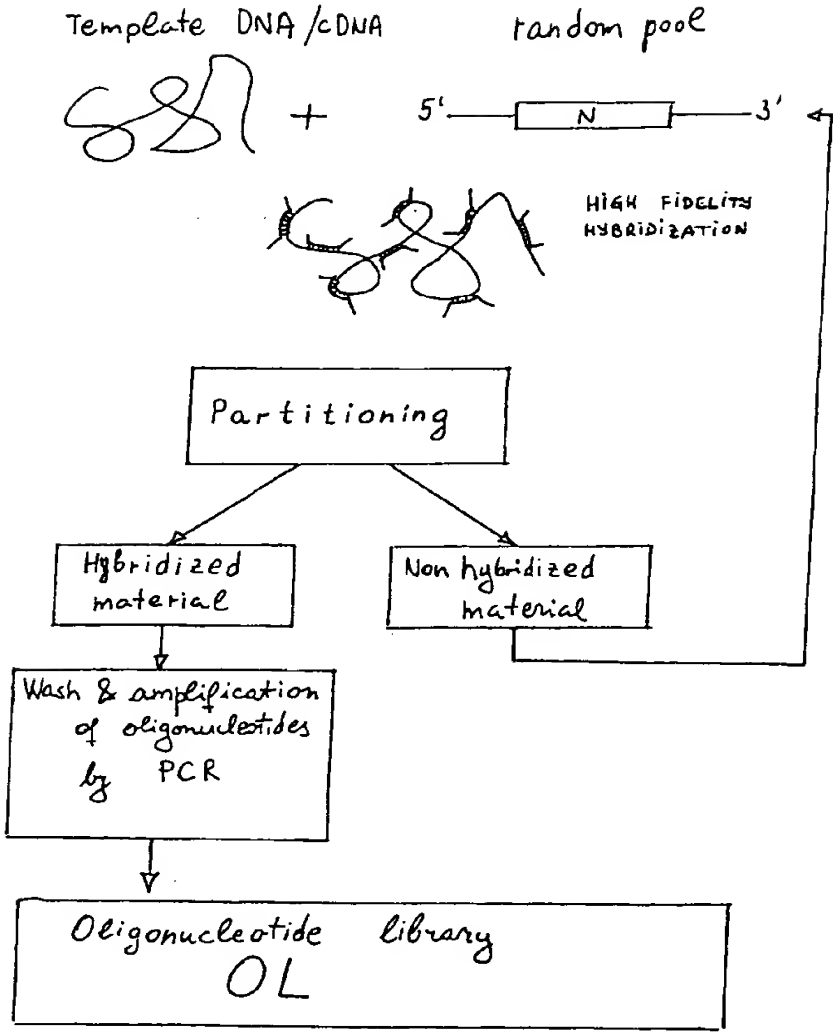

Template DNA/cDNA
random pool
5'
N
3'
HIGH FIDELITY HYBRIDIZATION
Partitioning
Hybridized material
Non hybridized material
Wash & amplification of oligonucleotides by PCR
Oligonucleotide library
OL

Fig. 1B)

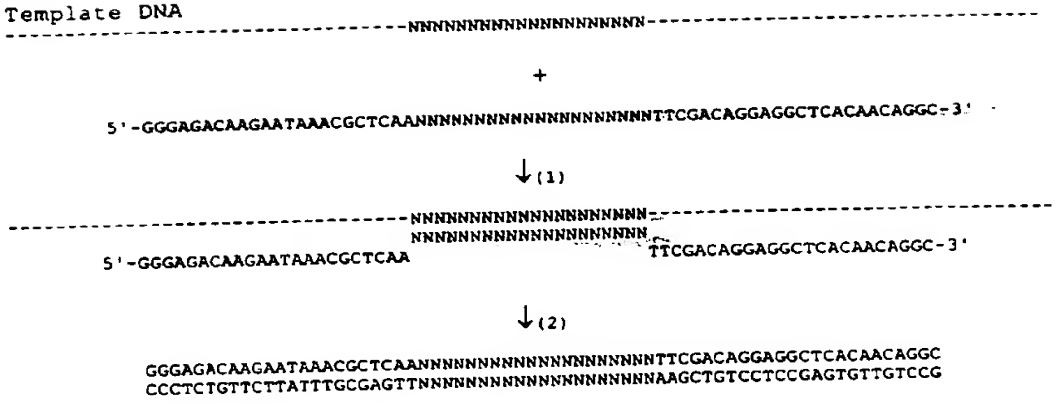

Template DNA
NNNNNNNNNNNNNNNNNNNN
+
5'-GGGAGACAAGAATAAACGCTCAANNNNNNNNNNNNNNNNNNNNTTCGACAGGAGGCTCACAACAGGC-3'
(1)
NNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNN
5'-GGGAGACAAGAATAAACGCTCAA
TTCGACAGGAGGCTCACAACAGGC-3'
(2)
GGGAGACAAGAATAAACGCTCAANNNNNNNNNNNNNNNNNNNNTTCGACAGGAGGCTCACAACAGGC
CCCTCTGTTCTTATTTGCGAGTTNNNNNNNNNNNNNNNNNNNNAAGCTGTCCTCCGAGTGTTGTCCG

Fig. 1C)

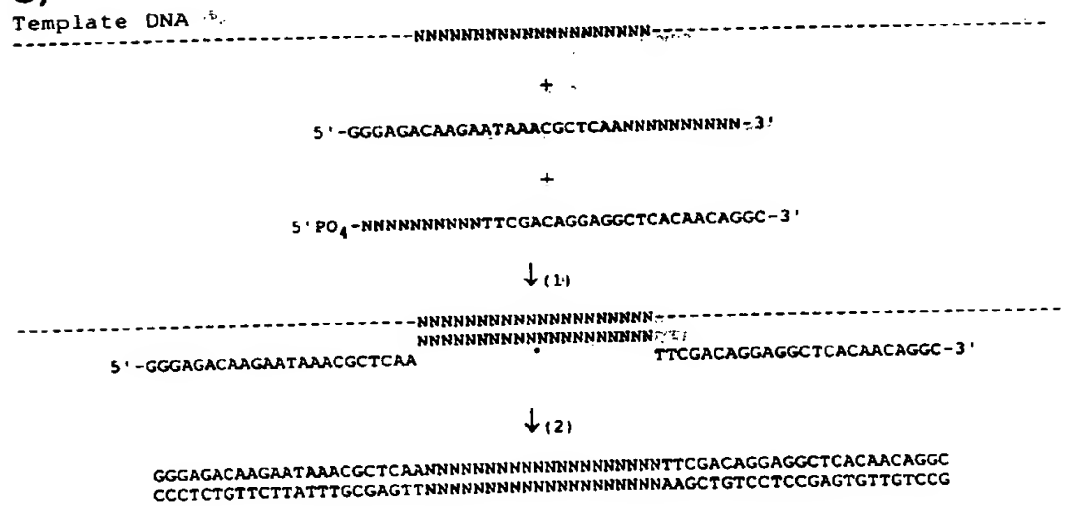

Template DNA
NNNNNNNNNNNNNNNNNNNN
+
5'-GGGAGACAAGAATAAACGCTCAANNNNNNNNNNN-3'
+
5'PO4-NNNNNNNNNNTTCGACAGGAGGCTCACAACAGGC-3'
(1)
NNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNN
5'-GGGAGACAAGAATAAACGCTCAA
TTCGACAGGAGGCTCACAACAGGC-3'
(2)
GGGAGACAAGAATAAACGCTCAANNNNNNNNNNNNNNNNNNNNTTCGACAGGAGGCTCACAACAGGC
CCCTCTGTTCTTATTTGCGAGTTNNNNNNNNNNNNNNNNNNNNAAGCTGTCCTCCGAGTGTTGTCCG

Fig. 2

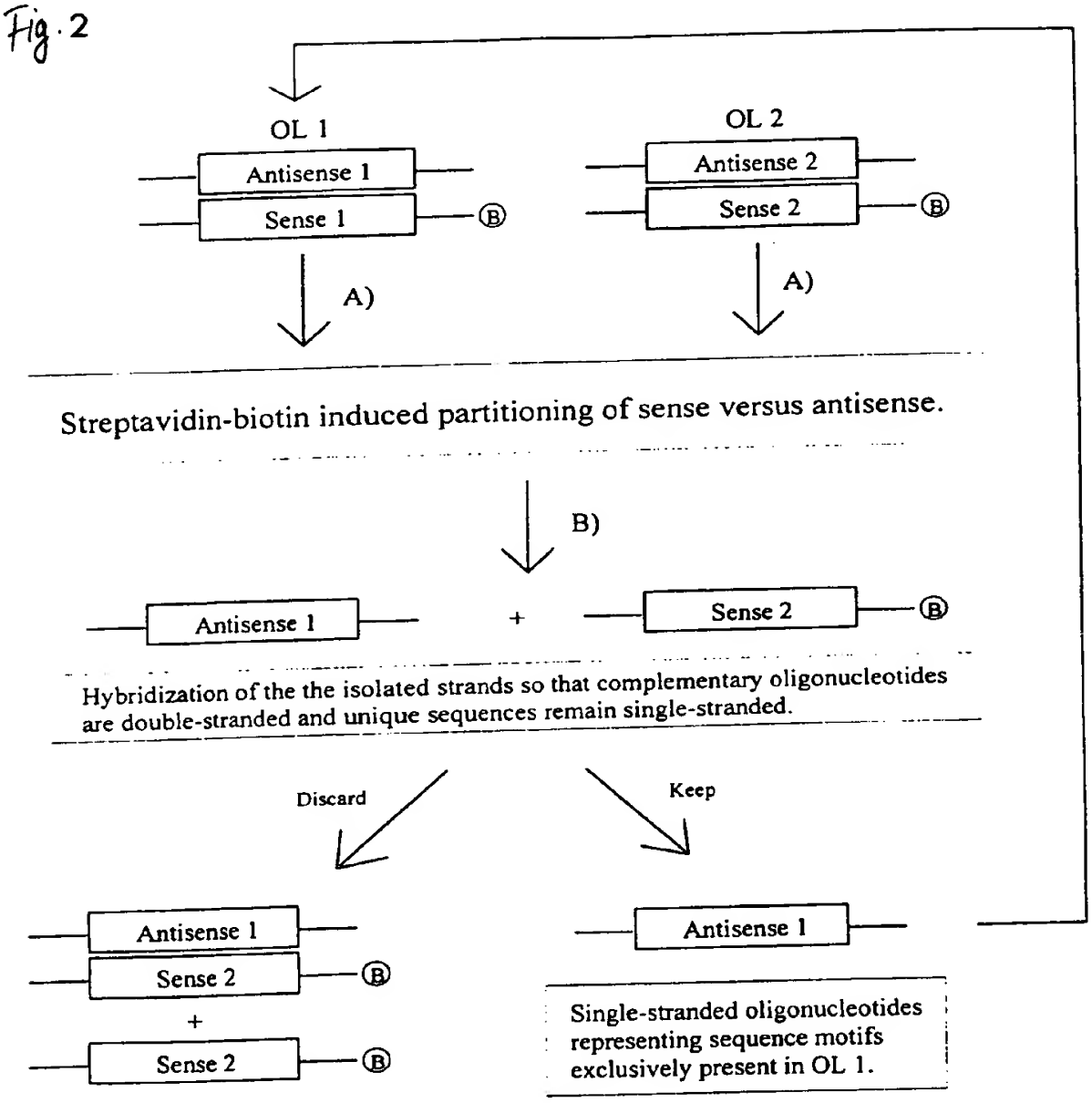
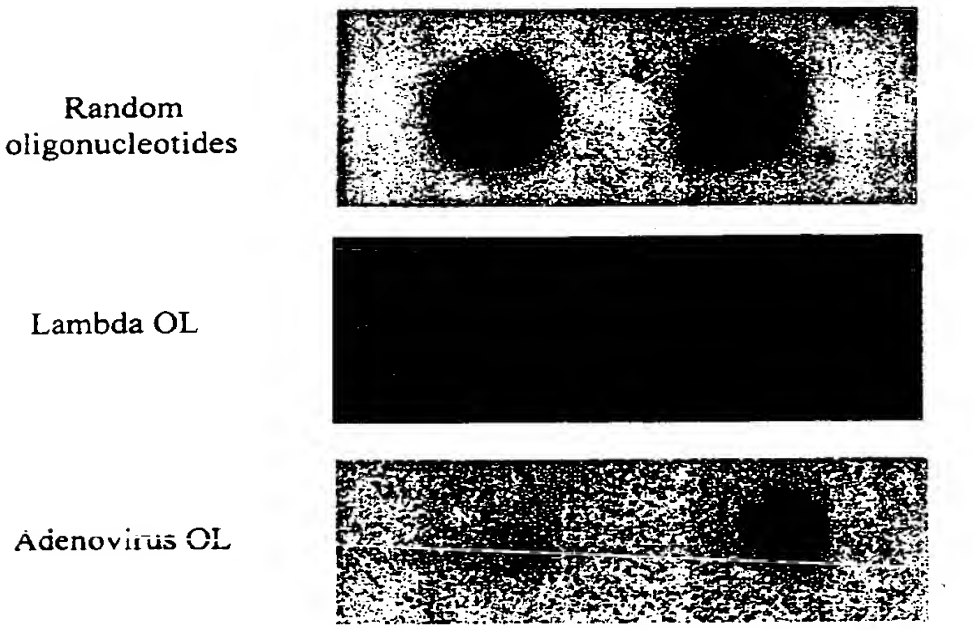

OL 1
Antisense 1
Sense 1
B
A)
OL 2
Antisense 2
Sense 2
B
A)
Streptavidin-biotin induced partitioning of sense versus antisense.
B)
Antisense 1
+
Sense 2
B
Hybridization of the the isolated strands so that complementary oligonucleotides are double-stranded and unique sequences remain single-stranded.
Discard
Keep
Antisense 1
Sense 2
B
+
Sense 2
B
Antisense 1
Single-stranded oligonucleotides representing sequence motifs exclusively present in OL 1.

Fig. 3


Probes
Target DNA
Lambda
Adenovirus
Random oligonucleotides
Lambda OL
Adenovirus OL